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Monitoring of wild birds for Newcastle disease virus in Switzerland using real time RT-PCR

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SHORT COMMUNICATIONS

MONITORING OF WILD BIRDS FOR NEWCASTLE DISEASE VIRUS IN SWITZERLAND USING REAL TIME RT-PCR

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ABSTRACT

Wild birds are considered to be the natural reservoir of avian paramyxovirus 1 (APMV-1), causing Newcastle disease, and are often suspected to be involved in outbreaks in domesticated birds. To assess the epidemiological status of wild birds living or overwintering in Switzerland, 3049 cloacal swabs covering the period 2003-2006 were screened for APMV-1 using real time RT-PCR. All samples were negative. This result seems in contrast with previously performed serological screenings of wild birds.

KEY WORDS

Newcastle disease, avian paramyxovirus 1, monitoring, wild bird, real time RT-PCR, Switzerland.

In 2006, Switzerland has been officially free from Newcastle disease (ND) for nine years, but continuous reports of isolated outbreaks from across Europe and especially from neighboring countries (Italy, 2000, 2003 and 2006; Austria, 2004; France, 2005 and 2006) (OIE, 2007) hint at the possibility of a future reappearance of ND. Three panzootics have been described in the last century (Alexander, 2001). However, closer genetic characterization and subtyping of the virus (lineages 1 to 6 with sublineages) shed some more light on the epidemiology and the evolution of the virus, suggesting a more complex and non-linear history of the disease, with essentially two independent but interacting host systems involved, wild bird populations (primordial reservoir) and poultry industry (secondary reservoir) (Aldous et al., 2003; Czeglédi et al., 2006). While most of the APMV-1 strains circulating in wild birds are lentogenic (Alexander, 2000; Globig et al., 2004), constant virus exchange between the two

reservoirs (where biosecurity conditions are poor) seems to be an important factor allowing new, potentially virulent strains to emerge (Takakuwa et al., 1998; Shengqing et al., 2002; Jørgensen et al., 2004). Outbreaks of ND in wild birds are very rare; in the last years mortality related to NDV was only reported in young cormorants in North America (Allison et al., 2005).

The mobility of migratory birds over long distances regardless of boundaries and frontiers and their population size – e.g. Switzerland is wintering place for 500'000 waterfowl alone - makes them an important and not easily controllable vector of APMV-1 dissemination (Zanetti et al., 2005). Another factor to consider is the enhancement of the wildlife/livestock interface caused by free-range management forms. This is particularly relevant for Swiss commercial poultry as an important share (> 40%) of the production is of this kind. Two screening projects were initiated in Switzerland over the last decade to assess the prevalence of APMV-1 in wild birds and poultry, both relying upon serological tests (Schelling et al., 1999; Wunderwald and Hoop, 2002). The objective of the present study was to verify the actual APMV-1 status in wild birds in Switzerland and thus to detect possible sources of NDV posing threats to domestic poultry.

Cloacal swabs from four groups of wild birds were collected between 2003 and 2006:

- Birds captured for ringing purposes in three relevant Swiss ornithologic places: Sempach lake (*n*=922), Klingnau dam reservoir (*n*=101) and Ulmet Höchi in the Jurassic hills (*n*=520), the first two stopover places for migrating waterfowl and the last a passage region for migratory songbirds).
- Birds shot during waterfowl hunting seasons in several Swiss cantons (*n*=306).
- Diseased or hurt birds from various sources (clinics, private animal shelters) (*n*=384) (Baumer, 2005).

- Birds found dead and collected during the extensive avian influenza monitoring campaign in winter 2005/2006 (*n*=816) (Dalessi et al., 2006).

90% of the samples was collected during the cold season (autumn - spring), and 50% of the birds tested were juveniles. 60% of the birds were apparently healthy (i.e. captured for ringing or shot by hunters), while the rest was found dead, diseased or hurt. Table 1 illustrates order, species and numbers of the birds tested.

The swabs were collected by ornithologists, hunters or veterinarians and sent to the laboratory within 24 hrs kept at +4 C. Swabs were put in phosphate-buffered saline immediately upon arrival at the laboratory or stored at –80 C until processing. In 2003/2004 swabs were pooled in groups of two or three in 1.5 ml phosphate-buffered saline, of which 750 microliters underwent further processing using the Ultraspec RNA isolation Kit (AMS Biotechnology, Lugano, CH) following the manufacturer's instructions. In 2005/2006 swabs were processed individually with the same amount of PBS using the RNeasy Mini Kit (Qiagen AG, Basel, CH) according to the instructions of the manufacturer. Extracted RNA samples were stored in RNAase free water at –80 C.

The samples were analyzed by a TaqMan one step realtime RT-PCR assay on an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, USA). Specific primer-probe sets for APMV-1 M (matrix) gene (for detection of all APMV-1 strains) and APMV-1 F (fusion) gene (for detection of mesogenic and velogenic APMV-1 strains) sequences were used as previously described and validated (Wise et al., 2004). The TaqMan probes for the matrix and fusion genes were labeled 5' Yakima-Yellow / 3' BHQ-1 and 5' FAM / 3' TAMRA, respectively.

The following volumes were used (totaling 30 µl per well): 15 µl of 2x Master Mix without UNG (Applied Biosystems), 0.75 µl of 40x MultiScribe and RNase Inhibitor Mix (Applied

Biosystems), 0.9 µl of each primer (10µM, final concentration 300 nM), 0.75 µl of probe (10 µM, final concentration 250 nM), 8.7 µl of RNAse free water and 3 µl of sample, previously heated to 94 C for 5 min and put on ice until preparation of the 96-well plate.

Thermal cycling conditions were set as follows: 30 min at 48 C for reverse transcription, 10 min at 95 C for RT deactivation and polymerase activation, and 55 cycles of 15 sec at 95 C for denaturation followed by 1 min at 60 C for annealing and extension.

Data from the thermocycler were analyzed with SDS Software (Applied Biosystems).

Several APMV-1 isolates (both velogenic and lentogenic strains), some of them from previous confirmed ND outbreaks in Switzerland, were used as positive controls on each assay plate. Furthermore isolates of other APMV serotypes were tested in order to check assay specificity (Table 2). A lower test sensitivity compared to virus isolation, probably due to the presence of PCR inhibitors in faeces, was reported (Wise et al., 2004; OIE, 2004). In order to estimate the relative sensitivity of the assay, a serial dilution of a sham inoculated fecal sample was comparatively tested with the standard virus isolation method (OIE, 2004): the RT-PCR detected 1 EID_{50}. The two methods seem to have comparable sensitivities.

All 3049 samples were negative for APMV-1 RNA. The last column of Table 1 illustrates the prevalence of APMV-1 in the Swiss population that can be excluded with the present sampling, calculated with the program FreeCalc (Cameron and Baldock, 1998).

This result is consistent with the negative APMV result of 200 routine egg cultivations of samples from wild birds performed in our lab between 2001 and 2005 (unpublished data). However, it seems in contrast with the serological screening projects recently carried out in Switzerland, where a seroprevalence for APMV of average 10% in wild birds was found (Schelling et al., 1999). The serological test used was not specific for APMV-1, so seroprevalence caused by other APMV serotypes cannot be ruled out. Considering that the

viral shedding period, and thus the diagnostic window of the RT-PCR method, is much shorter than the period of persistence of antibodies, a further explanation of this discrepancy could be a scenario of sporadic contact of migratory birds with virus strains of low virulence or vaccine strains abroad (ND-vaccination being prohibited in Switzerland) with subsequent occasional transmission into the non migrating bird population by birds still in the limited shedding period.

The samples used in this study originated from a plethora of sources and most were originally meant for avian influenza surveillance. The analogies in the epidemiology of AI and ND as well as the considerable amount of samples made the material suitable for this screening. However, in order to maximize its effectiveness, future monitoring should focus on collecting samples with the highest probability of virus detection. In addition to species (waterfowl and birds of prey showed highest seroprevalence in the serological screening), other epidemiological factors relative to APMV-1 in wild birds that must be considered have been indicated (Stallknecht et al., 1991): age (juvenile birds show higher prevalence) and season of sampling (prevalence seems to drop from September to December).

The M gene primer-probe set was designed to detect a broad spectrum of APMV-1 genotypes and was tested with wild bird samples (Wise et al., 2004); nonetheless, it was originally developed for virus detection in chicken and might not detect every genotype present in wildlife or might show varying sensitivity between bird species. This factor, in concomitance with an expected low overall prevalence of the virus, has to be carefully evaluated in interpreting the negative results, but has also to be balanced with the advantages of the method, namely speed and ease of sample collection and handling, in what seems to be the best compromise for a large population screening.

LITERATURE CITED

ALDOUS, E. W., MYNN, J. K., BANKS, J., AND D. J. ALEXANDER. 2003. A molecular epidemiological study of avian paramyxovirus type 1 (Newcastle disease virus) isolates by phylogenetic analysis of a partial nucleotide sequence of the fusion protein gene. Avian Pathology 32: 239-256.

ALEXANDER, D. J. 2000. Newcastle disease and other avian paramyxoviruses. Revue Scientifique et Technique – Office International des Epizooties 19: 443-462.

ALEXANDER, D. J. 2001. Gordon Memorial Lecture. Newcastle disease. British Poultry Science 42: 5-22.

ALLISON, A. B., GOTTDENKER, N. L., AND D. E. STALLKNECHT. 2005. Wintering of neurotropic velogenic Newcastle disease virus and West Nile virus in double-crested cormorants (*Phalacrocorax auritus*) from the Florida Keys. Avian Diseases 49: 292-297.

BAUMER, A. 2005. Aviäre Influenza: molekularbiologische Untersuchung von Wildvögeln und seroscreening von Wirtschaftsgeflügel in der Schweiz [Avian influenza: survey in wild birds and poultry in Switzerland using molecular and serological methods]. Doctoral Thesis, Vetsuisse faculty, University of Zurich, Switzerland, 29 pp.

CAMERON, A. R., AND F. C. BALDOCK. 1998. A new probability formula for surveys to substantiate freedom from disease. Preventive Veterinary Medicine 34(1):1-17.

CZEGLÉDI, A., UJVÁRI, D., SOMOGYI, E., WEHMANN, E., WERNER, O., AND B. LOMNICZI. 2006. Third genome size category of avian paramyxovirus serotype 1 (Newcastle disease virus) and evolutionary implications. Virus Research 120: 36-48.

DALESSI, S., HOOP, R. K., AND M. ENGELS. 2006. The 2005/2006 Avian Influenza Monitoring of Wild Birds and Commercial Poultry in Switzerland. Avian Diseases 51: 355–358.

GLOBIG, A., STARICK, E., AND O. WERNER. 2004. Untersuchung von Wildvögeln auf aviäre Influenza- und Paramyxoviren [Survey of avian influenza and paramyxoviruses in wild birds]. *In* Referatesammlung 67. Fachgespräch Geflügelkrankheiten, Hannover, Germany. DVG Service GmbH, Giessen, Germany, pp 73-82.

JØRGENSEN, P. H., HANDBERG, K. J., AHRENS, P., THERKILDSEN, O. R., MANVELL, R. J., AND D. J. ALEXANDER. 2004. Strains of avian paramyxovirus type 1 of low pathogenicity for chickens isolated from poultry and wild birds in Denmark. Veterinary Record 154: 497-500.

OIE. 2004. Newcastle disease. *In* Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. www.oie.int/eng/normes/mmanual/A_00038.htm. Accessed February 19, 2007.

OIE. 2007. Handistatus II. www.oie.int/hs2. Accessed February 19, 2007.

SCHELLING, E., THUR, B., GRIOT, C., AND L. AUDIGÉ. 1999. Epidemiological study of Newcastle disease in backyard poultry and wild bird populations in Switzerland. Avian Pathology 28: 263-272.

SHENGQING, Y., KISHIDA, N., ITO, H., KIDA, H., OTSUKI, K., KAWAOKA, Y., AND T. ITO. 2002. Generation of velogenic Newcastle disease viruses from a nonpathogenic waterfowl isolate by passaging in chickens. Virology 301: 206-211.

STALLKNECHT, D. E., SENNE D. A., ZWANK P. J., SHANE S. M., AND M.T. KEARNEY. 1991. Avian Paramyxoviruses from migrating and resident ducks in coastal Louisiana. Journal of Wildlife Diseases 27(1): 123-128.

TAKAKUWA, H., ITO, T., TAKADA, A., OKAZAKI, K., AND H. KIDA. 1998. Potentially virulent Newcastle disease viruses are maintained in migratory waterfowl populations. Japanese Journal of Veterinary Research 45: 207-215.

WISE, M. G., SUAREZ, D. L., SEAL, B. S., PEDERSEN, J. C., SENNE, D. A., KING, D. J.,

KAPCZYNSKI, D. R., AND E. SPACKMAN. 2004. Development of a real-time reverse-transcription PCR for detection of newcastle disease virus RNA in clinical samples. Journal of Clinical Microbiology 42: 329-338.

WUNDERWALD, C., AND R. K. HOOP. 2002. Serological monitoring of 40 Swiss fancy breed poultry flocks. Avian Pathology 31: 157-162.

ZANETTI, F., BERINSTEIN, A., PEREDA, A., TABOGA, O., AND E. CARRILLO. 2005. Molecular characterization and phylogenetic analysis of Newcastle disease virus isolates from healthy wild birds. Avian Diseases 49: 546-550.

Table 1. Order, species and numbers of the tested birds, NDV prevalence limit estimates.

Order	Species	Sampling 2003–04	Sampling 2005-06	Total	Excluded prevalence[a]
Anseriformes	Pochard *(Aythya farina)*	0	246	246	1.3%
	Tufted duck *(Aythya fuligula)*	11	243	254	1.2%
	Mute Swan *(Cygnus olor)*	15	131	146	2.1%
	Mallard *(Anas platyrhynchos)*	107	125	232	1.3%
	Duck, unidentified	66	106	172	1.8%
	Others	50	63	113	-
Gruiformes	Coot *(Fulica atra)*	19	202	221	1.4%
	Others	0	8	8	-
Pelicaniformes	Cormorant (*Phalacrocorax carbo sinensis*)	28	99	127	2.4%
Charadriiformes	Black-headed Gull *(Larus ridibundus)*	3	69	72	4.1%
	Yellow-legged Gull *(Larus cachinnans)*	0	22	22	12.8%
	Gull, unidentified	0	40	40	7.3%
	Others	0	3	3	-
Podicipediformes	Great crested grebe *(Podiceps cristatus)*	4	45	49	6%
	Little grebe *(Tachybaptus ruficollis)*	0	4	4	-
Passeriformes	Chaffinch *(Fringilla coelebs)*	318	211	529	0.6%
	Brambling *(Fringilla montifringilla)*	4	114	118	2.6%
	Great Tit *(Parus major)*	47	48	95	3.2%
	Song Thrush *(Turdus philomelos)*	23	36	59	5%
	Blackbird *(Turdus merula)*	31	42	73	4.1%
	Siskin *(Carduelis spinus)*	0	18	18	15.4%
	Others	107	90	197	-
Accipitriformes	Buzzard spp. *(Buteo sp.)*	13	24	37	7.8%
	Others	8	20	28	-
Other orders		110	76	186	-
Total		**964**	**2085**	**3049**	**0.1%**

[a] 95% confidence

Table 2. Reference strains, pathotype and primer-probe set specificity

Isolate	Pathotype	Matrix	Fusion
APMV-1/chicken/Switzerland/Safnern/95[a]	velogenic	+	+
APMV-1/chicken/Switzerland/Schaffhausen/96[a]	velogenic	+	+
APMV-1/chicken/Switzerland/H404/96[b]	lentogenic	+	-
PPMV-1/dove/Switzerland/T845/01	lentogenic	+	-
PPMV-1/dove/Switzerland/T32/93	lentogenic	+	-
APMV-1/chicken/NorthernIreland/Ulster/64	lentogenic	+	-
APMV-3/turkey/England/1087/82	not applicable	-	-
APMV-4/duck/Hongkong/D3/75	not applicable	-	-
APMV-7/dove/Tennessee/4/75	not applicable	-	-
APMV-8/goose/Delaware/1053/76	not applicable	-	-

[a] field cases with high fatality in poultry, [b] vaccine strain

ADDENDUM

As the article was submitted to the Journal of Wildlife Diseases as a short communication and thus kept in condensed form, certain aspects shall be further discussed here.

Stability of viral RNA in cloacal swabs

All samples were processed following to the guidelines of the OIE published in the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, which recommends storage of swabs at 4°C for up to 4 days after collection if immediate processing isn't possible.
Experience at the NRGK lab with mock infected swab samples and infected chorioallantoic fluid (CAF) is that viral RNA remains stable for weeks kept at +4°C.
Since in some of the tested samples Avian Influenza Virus RNA was detected (Dalessi et al., 2006), and given the similarities between the two viruses (both are negative-sense ssRNA viruses with similar structure), methodological errors in swab collection and processing, as well as RNA extraction can be excluded.
Moreover, Avian Influenza Virus in fecal samples shows no sign of inactivation even after 20 days of storage at +4°C (Lu et al., 2003), and APMV-1 has been reported to be able to survive in the environment (chicken manure at room temperature) for more than a week (Kinde et al., 2004). Further considering that for an RT-PCR assay the virions don't necessarily have to be infectious but only preserved RNA is needed, it is safe to assume that storing cloacal swabs for up to a few days at max +4°C before extraction doesn't affect the sensitivity of the assay.

Discrepancy between ND seropositive wild birds in 1996-7 and actual negative virus detection

The epidemiology of avian paramyxoviruses, in particular the dynamics of virus reservoirs in wildlife, is still poorly understood and involves complex interactions between environmental factors, (migratory) wild bird populations and poultry industry (Hanson et al., 2005).
The discrepancy between the presented results and those from the serological screenings can be approached at at least three levels.
First, since they were performed almost a decade apart, it could well be that the results simply reflect the effective decline of virus circulation in Europe in the last years. In the 1990's many outbreaks in commercial poultry premises were reported all across Europe, while in the early 2000's only scattered outbreaks of minor entity occurred (OIE, 2007).
Secondly, if the discrepancy lies within the methods used, further considerations are needed, on a) the interpretation of the results from different methods, as well as b) their specificity.

a) ND vaccination not being permitted in Switzerland, the seroprevalence detected in past years in wild birds can be explained in different ways, considering the differences in the length of the diagnostic windows of indirect (serological) and direct virus detection. First, contact between virus and the eventually seropositive birds could have happened in Switzerland, this would imply that some APMV strains are, if not endemic, at least sporadically circulating in the country. This scenario is directly rejected by the results of this work. A second hypothesis would see the contact between virus and migratory birds abroad. The limited viral shedding period (and thus detectability by direct methods) would be largely over by the time the bird returns, but seroconversion would be still measurable months thereafter. Testing this hypothesis

would imply an extensive sampling and recapturing birds during a whole migratory period in order to follow virus shedding and seroconversion directly.

b) The specificity of the ELISA assay used for the detection of antibodies against APMV-1 is known to be low compared to the EU- and OIE-reference test hemagglutination inhibition test (HI) (Baumer, 2005; Schelling et al., 1999). This is especially true when autolytic sera and tissue fluids are tested. In the study by Baumer only 3.5% of the ELISA positive samples resulted positive with HI.
Furthermore, performance of serological assays can significantly vary when applied to different species of birds than validated for (Gardner et al., 1996).
In contrast, this should be of minor importance in affecting the RT-PCR assay as the target is the viral RNA itself.

Thirdly, differential virus-host interactions have to be accounted for. While the RT-PCR assay used was designed to detect velogenic as well as lentogenic strains, infected wild birds might result negative because virus shedding in feces doesn't always occur, depending on strain pathogenicity (it is especially the case with lentogenic strains) and host immune response. On the other hand, wild waterbirds are considered the natural reservoir of (lentogenic) APMV-1, with populations harboring virus shedding healthy animals (Zanetti et al., 2005). The presence of such a reservoir would be detectable by RT-PCR analysis of cloacal swabs. Beside waterbirds, some species birds of prey also showed a high seroprevalence in Schelling's study (in particular sparrowhawks, with more than 50% seroprevalence), putatively as a result of feeding on infected birds. Conversely, Buzzards, sampled in relatively high numbers in the present study and the serological survey, and known to be susceptible to APMV-1, resulted negative to RT-PCR as well as seronegative in Schelling's study.
The number of samples from birds of prey available for this study being small, we tried in 2007 to increase the sampling size by collecting swabs from any sparrowhawk in treatment at at the Division of Zoo Animals and Exotic Pets of the Tierspital Zurich, which resulted in only three more samples, all of which resulted negative to RT-PCR.

Additional references (redundant references omitted)

LU, H., CASTRO, A. E., PENNICK, K., LIU, J., YANG, Q., DUNN, P., WEINSTOCK, D., AND D. HENZLER. 2003. Survival of Avian Influenza Virus H7N2 in SPF chickens and their environments. Avian diseases 47: 1015-1021.

KINDE, H., UTTERBACK, W., TAKESHITA, K., AND M. MCFARLAND. 2004. Survival of Exotic Newcastle Disease Virus in Commercial Poultry Environment Following Removal of Infected Chickens. Avian diseases 48: 669–674.

HANSON, B. A., SWAYNE, D. E., SENNE, D. A., LOBPRIES, D. S., HURST, J., AND D. E. STALLKNECHT. 2005. Avian Influenza Viruses and Paramyxoviruses in Wintering and Resident Ducks in Texas. Journal of Wildlife Diseases 41(3): 624–628.

GARDNER, I. A., HIETALA, S., AND W. M. BOYCE. 1996. Validity of using serological tests for diagnosis of diseases in wild animals. Revue scientifique et technique de l'office international des epizooties 15: 323-335.

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